The Patent Office

The Patent Office
Cardiff Road
Newport
Gwent
NP9 1RH

08/436265

08/436,265

I, the undersigned, being an officer duly authorised in accordance with Section 62(3) of the Patents and Designs Act 1907, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the Patent application identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or the inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.

Signed W. Russell

Dated 17 JAN 1994

OC1

PRIORITY DOCUMENTS

9321344.5
15 Oct. 1993

For official use

15 OCT 1993

Your reference 70/4201/01

9321344.5

Notes
Please type, or write in dark ink using CAPITAL letters. A prescribed fee is payable for a request for grant of a patent For details, please contact the Patent Office (telephone 071-438 4700).
Rule 16 of the Patents Rules 1990 is the main rule governing the completion and filing of this form

The Patent Office **Request for grant of a Patent Form 1/77** **Patents Act 1977**

1 Title of invention

1 Please give the title of the invention PROTEIN KINASES

2 Applicant's details

Do not give trading styles, for example, 'Trading as XYZ company', nationality or former names, for example, 'formerly (known as) ABC Ltd' as these are not required.

☐ **First or only applicant**

2a If you are applying as a corporate body please give:

Corporate name Ludwig Institute for Cancer Research

Country *(and State of incorporation, if appropriate)* United Kingdom

2b If you are applying as an individual or one of a partnership please give in full:

Surname

Forenames

2c **In all cases, please give the following details:**

Address St. Mary's Hospital Medical School
Norfolk Place
Paddington
London

UK postcode *(if applicable)* W2 1PG

Country England

ADP number *(if known)*

Warning
After an application for a Patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977 and will inform the applicant if such prohibition or restriction is necessary. Applicants resident in the United Kingdom are also reminded that under Section 23, applications may not be filed abroad without written permission unless an application has been filed not less than 6 weeks previously in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction revoked.

2d, 2e and 2f: If there are further applicants please provide details on a separate sheet of paper.

☐ **Second applicant (if any)**

2d If you are applying as a corporate body please give:
Corporate name

Country *(and State of incorporation, if appropriate)*

2e If you are applying as an individual or one of a partnership please give in full:

Surname

Forenames

2f **In all cases, please give the following details:**

Address

UK postcode
(if applicable)

Country
ADP number
(if known)

3 An address for service in the United Kingdom must be supplied

Please mark correct box

3 Address for service details

3a Have you appointed an agent to deal with your application?

Yes [X] No [ ] ⇒ **go to 3b**

⇓

please give details below

Agent's name

GILL JENNINGS & EVERY

Agent's address

Broadgate House
7 Eldon Street
London

Postcode EC2M 7LH

Agent's ADP number 745002

3b: If you have appointed an agent, all correspondence concerning your application will be sent to the agent's United Kingdom address.

3b If you have not appointed an agent please give a name and address in the United Kingdom to which all correspondence will be sent:

Name

Address

Postcode

Daytime telephone number *(if available)*

ADP number
(if known)

4 Reference number

4 Agent's or applicant's reference number *(if applicable)* 70/4201/01

5 Claiming an earlier application date

5 Are you claiming that this application be treated as having been filed on the date of filing of an earlier application?

Please mark correct box

Yes ☐ No ☒ ⇨ go to 6

⇓

please give details below

☐ number of earlier application or patent number

☐ filing date

(day month year)

☐ and the Section of the Patents Act 1977 under which you are claiming:

Please mark correct box

15(4) (Divisional) ☐ 8(3) ☐ 12(6) ☐ 37(4) ☐

6 Declaration of priority

6 If you are declaring priority from a PCT application please enter 'PCT' as the country and enter the country code (for example, GB) as part of the application number.

6 If you are declaring priority from previous application(s), please give:

Please give the date in all number format, for example, 31/05/90 for 31 May 1990.

Country of filing	Priority application number *(if known)*	Filing date *(day, month, year)*

7 The answer must be 'No' if

- any applicant is not an inventor
- there is an inventor who is not an applicant, **or**
- any applicant is a corporate body.

7 Inventorship

7 Are you (the applicant or applicants) the sole inventor or the joint inventors?

Please mark correct box

Yes ☐ No ☒ ➡ **A Statement of Inventorship on Patents Form 7/77 will need to be filed (see Rule 15)**

8 Please supply duplicates of claim(s), abstract, description and drawing(s).

8 Checklist

8a Please fill in the number of sheets for each of the following types of document contained in this application.

Continuation sheets for this Patents Form 1/77	0		
Claim(s)	0	Description	15
Abstract	0	Drawing(s)	5

8b Which of the following documents also accompanies the application?

Please mark correct box(es)

Priority documents *(please state how many)*	0
Translation(s) of Priority documents *(please state how many)*	0
Patents Form 7/77 - Statement of Inventorship and Right to Grant *(please state how many)*	0
Patents Form 9/77 - Preliminary Examination/Search	
Patents Form 10/77 - Request for Substantive Examination	

9 You or your appointed agent (see Rule 90 of the Patents Rules 1990) must sign this request.

9 Request

I/We request the grant of a patent on the basis of this application.

For the Applicant
GILL JENNINGS & EVERY

Please sign here ⇒

Signed *R.E. Perry* Date 14 October 1993 (day month year)

R.E. Perry

A completed fee sheet should preferably accompany the fee.

Please return the completed form, attachments and duplicates where requested, together with the prescribed fee to either:

☐ **The Comptroller**
The Patent Office
Cardiff Road
Newport
Gwent
NP9 1RH

or

☐ **The Comptroller**
The Patent Office
25 Southampton Buildings
London
WC2A 1AY

PROTEIN KINASES

This invention is related to the one or more inventions described in British Patent Applications Nos. 9224057.1, filed 17th November 1992; 9304677.9 and 9304680.3, both filed 8th March 1993; 9311047.6, filed 28th May 1993; 9313763.6, filed 2nd July 1993; and 9316099.2, filed 3rd August 1993. In particular, this invention relates to nucleotides, proteins obtained by expression therefrom and antibodies raised to peptides derived from the sequence, e.g. by the means described in the Application No. 9304680.3.

The earlier Applications relate, inter alia, to kinases denoted ALK-1 to ALK-6. The complete cDNA sequences for mouse ALK-1 and ALK-4 are included herein (see pages 14 and 15). Mouse ALK-1 (clone name AM6 with 1.9 kb insert) was obtained from a mouse placenta λZAP II cDNA library (obtained from Hideo Toyoshima), using human ALK-1 cDNA as a probe. Mouse ALK-4 (clone 8a1 with 2.3 kb insert) was also obtained from the mouse placenta λZAP II cDNA library, using human ALK-4 cDNA library as a probe.

Transforming growth factor (TGF)-β and activin exert their cellular effects by forming heteromeric complexes of type I (53 kDa) and type II (80 kDa) receptors[1-3]. The TGF-β type I receptor cannot bind ligand in the absence of the type II receptor, and the TGF-β type II receptor cannot transduce signals without the type I receptor[4-7]. The type II receptors for TGF-β (TβR-II)[8] and activin (ActR-II[9] and ActR-IIB[10,11]) are serine/threonine kinases. Moreover, we[12,13] and others[14-17] have recently identified a series of serine/threonine kinase receptors of sizes corresponding to type I receptors. Of these receptors, denoted activin receptor-like kinase (ALK)-1 to -6 by us, ALK-5 has been shown to be a functional TGF-β type I receptor[13]. Here, we have systematically investigated the abilities of ALKs to serve as type I receptors for TGF-β and activin. Our results revealed that ALKs can form heteromeric complexes with TβR-II and ActR-II after co-transfection into COS cells; however, only ALK-5 is a functional TGF-β type I receptor with regard to induction of plasminogen activator inhibitor (PAI)-1, and ALK-2 and ALK-4 are high affinity activin type I receptors.

We have previously identified five novel serine/threonine kinase receptors, termed ALK-1 to -5[12,13]. A sixth clone termed ALK-6 was obtained by screening a 12 day mouse embryo cDNA library using a probe from a part of the kinase domain of ALK-4 under low stringency hybridization conditions (Fig. 1a). A typical hydrophobic leader sequence is not observed in the N-terminus of the translated region; however, the ALK-6 protein is efficiently expressed at the cell surface (see below). Northern blot analysis revealed a limited expression profile: a transcript of 7.2 kb was found in mRNA from the brain and a very weak hybridization was seen with mRNA from the lung (Fig. 1b). Mouse ALK-6 is most similar to human ALK-3, but cloning and sequence analysis of mouse cDNA for ALK-3 (our unpublished data) indicate that ALK-6 is a novel serine/threonine kinase receptor. A phylogenetic tree based on the similarities between the kinase domains of ALKs and other mammalian serine/threonine kinase receptors[8-11] is

shown in Fig. 1c. ALKs are more similar to each other than to the TGF-β and activin type II receptors. The calculated molecular weights of ALK-1 to -6 are 53,600-57,500, *i.e.* smaller than those of the type II receptors, and similar to the reported sizes for type I receptors[9,18]. Moreover, ALK-2 (Tsk-7L)[14] and ALK-5[13] have recently been shown to form heteromeric complexes with TβR-II and to bind TGF-β. We therefore systematically investigated which ALKs can act as type I receptors for TGF-β and activin.

Affinity cross-linking studies using ^{125}I-TGF-β1 revealed that COS-1 cells express low or not detectable levels of TGF-β type I or type II receptors (data not shown). Transfection of cDNAs for ALKs into COS-1 cells did not show any appreciable binding of ^{125}I-TGF-β1 (data not shown), consistent with the previous observation that type I receptors do not bind TGF-β in the absence of type II receptors[4-6]. When the TβR-II cDNA was co-transfected with cDNAs for the different ALKs, type I receptor-like complexes were seen, at different levels, in each case. COS-1 cells transfected with TβR-II and ALK cDNAs were analyzed by affinity cross-linking followed by immunoprecipitation using specific antisera against TβR-II (Fig. 2a) or ALKs (Fig. 2b). Each one of the ALKs bound ^{125}I-TGF-β1 and was coimmunoprecipitated with the TβR-II complex using an antiserum against TβR-II (Fig. 2a). Comparison of the efficiency of the different ALKs to form heteromeric complexes with TβR-II, revealed that ALK-5 formed such complexes more efficiently than the other ALKs. The size of the cross-linked complex was larger for ALK-3 than for other ALKs, consistent with its slightly larger size (Fig. 1c). When the cross-linked complexes were immunoprecipitated by antibodies specific for the different ALKs, each one of the ALKs was immunoprecipitated in complex with TβR-II (Fig. 2b). Also in this analysis, ALK-5 formed a heteromeric complex with TβR-II more efficiently than the other ALKs.

Two different approaches were used to elucidate which ALKs are physiological type I receptors for TGF-β. First, we investigated which ALKs serve as TGF-β type I receptors in non-transfected, TGF-β-responsive cell lines. Several different cell lines were affinity labeled with ^{125}I-TGF-β1, cross-linked and immunoprecipitated by antisera

against different ALKs. Only the antisera against ALK-5 efficiently immunoprecipitated the cross-linked type I and type II receptor complexes in a mink lung epithelial cell line (Mv1Lu), porcine aortic endothelial (PAE) cells (Fig. 2c) and human foreskin fibroblasts (data not shown).

We next investigated whether ALKs restore the responsiveness to TGF-β in the R mutant of Mv1Lu cells, which lack ligand-binding ability of the TGF-β type I receptor, but have intact type II receptor[4]. The R mutant cells were transfected with the cDNA for ALKs or a control plasmid, and tested for the production of PAI-1 after the addition of TGF-β1. As we have previously reported[13], the wild type mink cells and the R mutant cells transfected with the ALK-5 cDNA responded to TGF-β1, and produced a characteristic 45 kDa PAI-1 protein in the extracellular matrix (Fig. 2d). In contrast, the R mutant cells that were transfected with other ALKs did not produce PAI-1 upon the addition of TGF-β1. Thus, only ALK-5 was able to form a signalling TGF-β receptor complex with regard to PAI-1 induction; we therefore suggest that it should be named TβR-I.

Using similar approaches as those described above for the identification of TGF-β binding ALKs, we then investigated whether ALKs bind activin in the presence of ActR-II. COS-1 cells were transfected with the cDNAs for ALKs and ActR-II, affinity labeled and cross-linked with ^{125}I-activin A. All ALKs appear to bind activin A in the presence of ActR-II (Fig. 3a). This could be more clearly demonstrated by affinity cross-linking followed by immunoprecipitation using antisera against ActR-II or ALKs. ALK-2 and ALK-4 bound ^{125}I-activin A and coimmunoprecipitated with ActR-II by the antiserum against ActR-II or ALKs (Fig. 3b). Other ALKs also bound ^{125}I-activin A, but with lower efficiency compared to ALK-2 and ALK-4.

In order to investigate whether ALKs are physiological activin type I receptors, we attempted to identify endogenous activin type I receptors expressed in activin-responsive cells. Mv1Lu cells as well as the R mutant express both type I and type II receptors for activin, and the R mutant cells produce PAI-1 upon the addition of activin A (data not shown). Mv1Lu cells were labeled with ^{125}I-activin A, cross-linked and

immunoprecipitated by the antisera against ActR-II or ALKs. The type I and type II receptor complexes in Mv1Lu cells were immunoprecipitated only by the antisera against ALK-2, ALK-4 and ActR-II (Fig. 3c). Similar results were obtained by the R mutant cells (data not shown). PAE cells do not bind activin because of the lack of type II receptors for activin; however, after transfection of a chimeric receptor containing the extracellular domain and the C-terminal tail of ActR-II and the kinase domain of TβR-II, the cells (PAE/Chim A) bound ^{125}I-activin A and were growth inhibited by the addition of activin A (our unpublished data). Similar to Mv1Lu cells, activin type I receptor complexes in PAE/Chim A cells were immunoprecipitated by the ALK-2 and ALK-4 antisera (Fig. 3c). These results indicate that both ALK-2 and ALK-4 act as physiological type I receptors for activin in these cells.

There are no known established cell lines that lack type I receptors or both type I and type II receptors for activin. If there are two types of ActR-I, *i.e.* ALK-2 and ALK-4, which are widely expressed[12], such cell types might be difficult to find. We, therefore, could not study the restoration of activin signals by the transfection of ALKs. After submission of this manuscript, Attisano *et al.*[16] reported the cDNA cloning of ALK-1 (TSR-I) and ALK-2 (ActR-I) and binding of activin to both of them. Moreover, using the R mutant clone R1B, they showed that ALK-2, in combination with ActR-II, transduced an activin-induced transcriptional response. We found that the R mutant clone 4-2 bound activin A and produced PAI-1 upon stimulation with activin A without transfection of ALKs or ActR-II cDNA (data not shown). Since both ALK-2 and ALK-4 served as high affinity type I receptors for activin A in Mv1Lu cells and the PAE/Chim A cells, we suggest to term them ActR-IA and ActR-IB, respectively.

The binding of TGF-β1 and activin A to the different ALKs is schematically illustrated in Fig. 4. There exists no cross-binding between TGF-β and activin to the type II receptors[9,10]. In contrast, binding of the ligands to type I receptors (ALKs) are less strict, and they can bind TGF-β1 and activin in the presence of the respective type II receptors. The ALKs which are most similar in their structures, do not necessarily bind the same ligands. For example, ALK-4/ActR-IB and ALK-5/TβR-I are highly similar to

References

1. Massagué, J. *Cell* 69, 1067-1070 (1992).
2. Lin, H.Y. & Lodish, H.F. *Trends Cell Biol.* 3, 14-19 (1993).
3. Miyazono, K., ten Dijke, P., Ichijo, H. & Heldin, C.-H. *Adv. Immunol.* in press (1994).
4. Laiho, M., Weis, F.M.B. & Massagué, J. *J. Biol. Chem.* 265, 18518-18524 (1990).
5. Laiho, M., Weis, F.M.B., Boyd, F.T., Ignotz, R.A. & Massagué, J. *J. Biol. Chem.* 266, 9108-9112 (1991).
6. Wrana, J.L., Attisano, L., Cárcamo, J., Zentella, A., Doody, J., Laiho, M., Wang, X.-F. & Massagué, J. *Cell* 71, 1003-1014 (1992).
7. Inagaki, M., Moustakas, A., Lin, H.Y., Lodish, H.F. & Carr, B.I. *Proc. Natl. Acad. Sci. U.S.A.* 90, 5359-5363 (1993).
8. Lin, H.Y., Wang, X.-F., Ng-Eaton, E., Weinberg, R.A. & Lodish, H.F. *Cell* 68, 775-785 (1992).
9. Mathews, L.S. & Vale, W.W. *Cell* 65, 973-982 (1991).
10. Attisano, L., Wrana, J.L., Cheifetz, S. & Massagué, J. *Cell* 68, 97-108 (1992).
11. Mathews, L.S., Vale, W.W. & Kintner, C.R. *Science* 255, 1702-1705 (1992).
12. ten Dijke, P., Ichijo, H., Franzén, P., Schulz, P., Saras, J., Toyoshima, H., Heldin, C.-H. & Miyazono, K. *Oncogene* 8, 2879-2887 (1993).
13. Franzén, P., ten Dijke, P., Ichijo, H., Yamashita, H., Schulz, P., Heldin, C.-H. & Miyazono, K. *Cell* in press (1993).
14. Ebner, R., Chen, R.-H., Shum, L., Lawler, S., Zioncheck, T.F., Lee, A., Lopez, A.R. & Derynck, R. *Science* 260, 1344-1348 (1993).
15. Matsuzaki, K., Xu, J., Wang, F., McKeehan, W.L., Krummen, L. & Kan, M. *J. Biol. Chem.* 268, 12719-12723 (1993).
16. Attisano, L., Cárcamo, J., Ventura, F., Weis, F.M.B., Massagué, J. & Wrana, J.L. *Cell* in press (1993).

17. He, W.W., Gustafson, M.L., Hirobe, S. & Donahoe, P.K. *Dev. Dyn.* 196, 133-142 (1993).

18. Cheifetz, S., Andres, J.L. & Massagué, J. *J. Biol. Chem.* 263, 16984-16991 (1988).

19. von Heijne, G. *Nucl. Acids Res.* 14, 4683-4690 (1986).

20. Kanzaki, T., Olofsson, A., Morén, A., Wernstedt, C., Hellman, U., Miyazono, K., Claesson-Welsh, L. & Heldin, C.-H. *Cell* 61, 1051-1061 (1990).

21. Palazzolo, M.J. *et al. Gene* 88, 25-36 (1990).

22. Sanger, F., Nicklen, S. & Coulson, A.R. *Proc. Natl. Acad. Sci. U.S.A.* 74, 5463-5467 (1977).

23. Laiho, M., Rönnstrand, L., Heino, J., DeCaprio, J.A., Ludlow, J.W., Livingston, D.M. & Massagué, J. *Mol. Cell. Biol.* 11, 972-978 (1991).

24. Truett, M.A. *et al. DNA* 4, 333-349 (1985).

25. Frolik, C.A., Wakefield, L.M., Smith, D.M. & Sporn, M.B. *J. Biol. Chem.* 259, 10995-11000 (1984).

26. Biobel, G. & Dobberstein, B. *J. Cell Biol.* 67, 835-851 (1975).

27. Gullick, W.J., Downward, J. & Waterfield, M.D. *EMBO J.* 4, 2869-2877 (1985).

28. Hammacher, A., Hellman, U., Johnsson, A., Östman, A., Gunnarson, K., Westermark, B., Wasteson, A. & Heldin, C.-H. *J. Biol. Chem.* 263, 16493-16498 (1988).

29. Ichijo, H., Yamashita, H., ten Dijke, P., Eto, Y., Heldin, C.-H. & Miyazono, K. *Biochem. Biophys. Res. Commun.* 194, 1508-1514 (1993).

ACKNOWLEDGEMENTS. We thank Y. Eto (Ajinomoto Co., Inc., Japan) for providing recombinant human activin A, H. Ohushi (Kirin Brewery Co., Ltd, Japan) for recombinant human TGF-β1, Kari Alitalo for a mouse cDNA library, L. Mathews and W.W. Vale for mouse ActR-II cDNA, and Danny Huylebroeck for valuable discussion. We also thank Anita Morén and Susanne Grimsby for technical assistance, and Ulla

Engström and Christer Wernstedt for preparing the synthetic peptides and oligonucleotides, respectively. Nucleotide sequence of mouse ALK-6 is deposited in EMBL/GenBank data library (accession number Z23143).

and two times for 30 min with 0.3 x SSC, 0.1% SDS. The filter was then subjected to autoradiography.

FIG. 2. Binding of TGF-β1 to ALKs *(a-c)* and transduction of a TGF-β signal by ALKs in the TGF-β type I receptor deficient cells *(d)*. *a* and *b*, COS-1 cells were transfected with cDNAs for TβR-II and ALKs, and affinity labeled with ^{125}I-TGF-β1 in the presence or absence of excess unlabeled TGF-β1 (cold TGF-β1), followed by cross-linking and immunoprecipitation using the antisera against TβR-II *(a)* or ALKs *(b)*. The TβR-II antiserum was used for the ALK cDNA (-) in *(b)*. Each lane in *(b)* was analyzed in the same gel and subjected to exposure for an equally long time. *c*, Identification of the TGF-β type I receptor complex on Mv1Lu cells and PAE cells. The cells were affinity labeled with ^{125}I-TGF-β1 and cross-linked, followed by immunoprecipitation using antisera specific for ALKs. The cross-linked complexes from PAE cells were also subjected to immunoprecipitation using the TβR-II antiserum. *d*, TGF-β induced PAI-1 production was tested in wild type (WT) Mv1Lu cells or in the R mutant cells after transfection of cDNAs for ALKs. PAI-1 was observed as a characteristic 45 kDa band[23]. METHODS. Transient expression plasmids of ALK-1 to -6 and TβR-II were generated by subcloning into the pSV7d expression vector[24] or into the pcDNA I expression vector (Invitrogen). For transient transfection, COS-1 cells (American Type Culture Collection) were transfected with 10 μg each of plasmids by a calcium phosphate precipitation method using a mammalian transfection kit (Stratagene), following the manufacturer's protocol. Recombinant human TGF-β1 was iodinated using the chloramine T method[25]. Cross-linking and immunoprecipitation were performed as previously described[13]. The samples were analyzed by SDS-gel electrophoresis[26] and autoradiography. Rabbit antisera against ALK-5 and TβR-II were made against the intracellular juxtamembrane part of ALK-5 and the C-terminal part of TβR-II, respectively, as previously reported[13]. Antisera against ALK-1, 2, 3, 4, and 6 were raised against synthetic peptides corresponding to the amino acid sequences of the intracellular juxtamembrane parts of ALKs; ALK-1 peptide, R(145)RQEK QRGLH SELGE SSLIL KA; ALK-2 peptide,

R(151)RNQE RLNPR DVEYC TIEGL IT; ALK-3 peptide, K(181)SISS RRRYN RDLEQ DEAFI PV; ALK-4 peptide, Q(153)RVYH NRQRL DMEDP SCEM; ALK-6 peptide, K(151)RQEA RPRYS IGLEQ DET. The synthetic peptides were coupled to keyhole limpet hemocyanin (Calbiochem-Behring) using glutaraldehyde[27]. The coupled peptides were mixed with Freund's adjuvant and used to immunize rabbits[28]. For PAI-1 assay, the R mutant of Mv1Lu cells (clone 4-2) were transfected with 10 μg of plasmids containing cDNAs for ALKs or a control plasmid (ALK cDNA - in the R mutant) by the calcium phosphate precipitation method. Transfected cells were incubated with or without 16 ng/ml of TGF-β1 for 2 h in serum-free MCDB 104 without methionine, and then labeled with [^{35}S]methionine (40 μCi/ml) for 2 h. Extracellular matrix proteins were prepared as described previously[23], and analyzed by SDS-gel electrophoresis using 8% polyacrylamide gels followed by fluorography using Amplify (Amersham).

FIG. 3. Identification of activin type I receptors. *a*, COS-1 cells were co-transfected with cDNAs for ALKs and ActR-II and analyzed for binding and cross-linking of ^{125}I-activin A in the presence or absence of excess unlabelled activin A (cold activin A). *b*, The cross-linked complexes were subjected to immunoprecipitation using antisera against ActR-II or ALKs. *c*, Binding and cross-linking of ^{125}I-activin A to Mv1Lu cells and PAE/Chim A cells were analyzed before (antiserum -) or after immunoprecipitation using antibodies specific for ALKs. Antisera used in *(b)* and *(c)* are shown as: II, ActR-II antiserum; 1 to 6; ALK-1 to -6 antisera.

METHODS. Transient expression plasmids of ActR-II were generated by subcloning into the pSV7d vector[29]. An antiserum against ActR-II was prepared against the C-terminal part of the ActR-II protein[29]. Recombinant human activin A was iodinated using the chloramine T method[9]. Autoradiographies were analyzed by PhosphorImager (Molecular Dynamics). For the generation of PAE/Chim A cells, a plasmid (chim A) containing the extracellular domain and C-terminal tail of ActR-II (amino acids -19 to 116 and 465 to 494, respectively, according to Ref. 9) and the kinase domain of TβR-II (amino acids 160 to 543 according to Ref. 8) was constructed and transferred into pcDNA I/neo

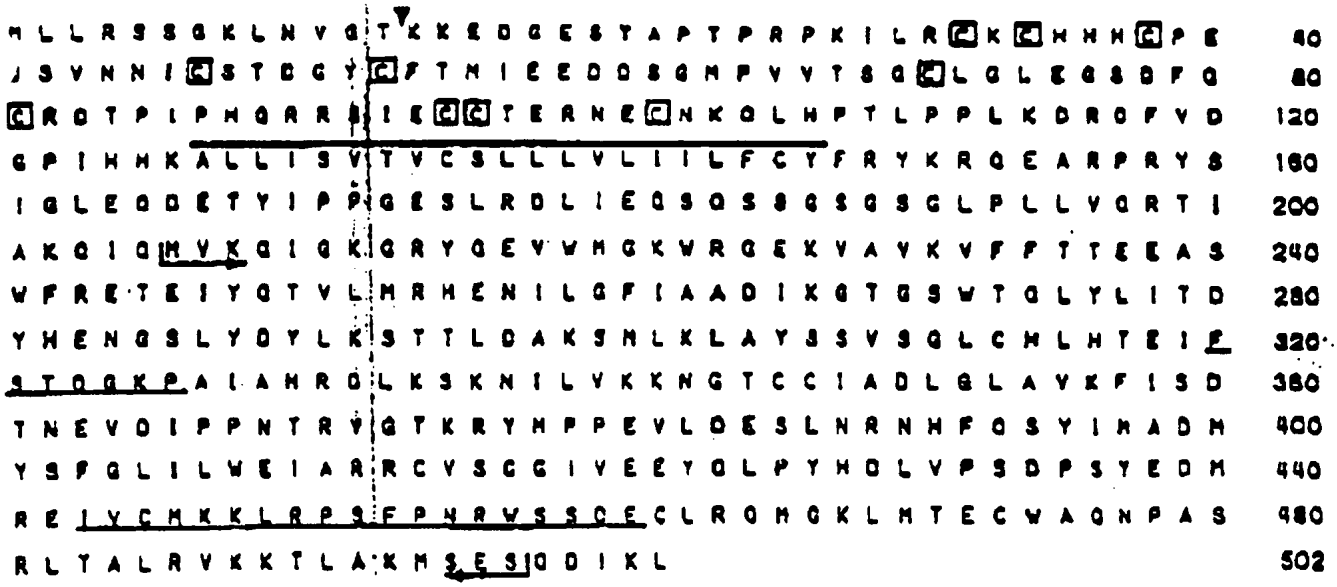
(Invitrogen). PAE cells were stably transfected with the chim A plasmid by electroporation, and cells expressing the chim A protein were established as described[13].

FIG. 4. Schematic illustration of the binding of TGF-β and activin to ALKs. TGF-β can bind all ALKs in the presence of TβR-II, but only ALK-5/TβR-I binds TGF-β efficiently and transduces a TGF-β signal (PAI-1 induction). Activin can also bind all ALKs in the presence of ActR-II, but only ALK-2/ActR-IA and ALK-4/ActR-IB efficiently form complexes with ActR-II in activin-responsive cells. ALK-2/ActR-IA was recently shown to transduce an activin signal[16].

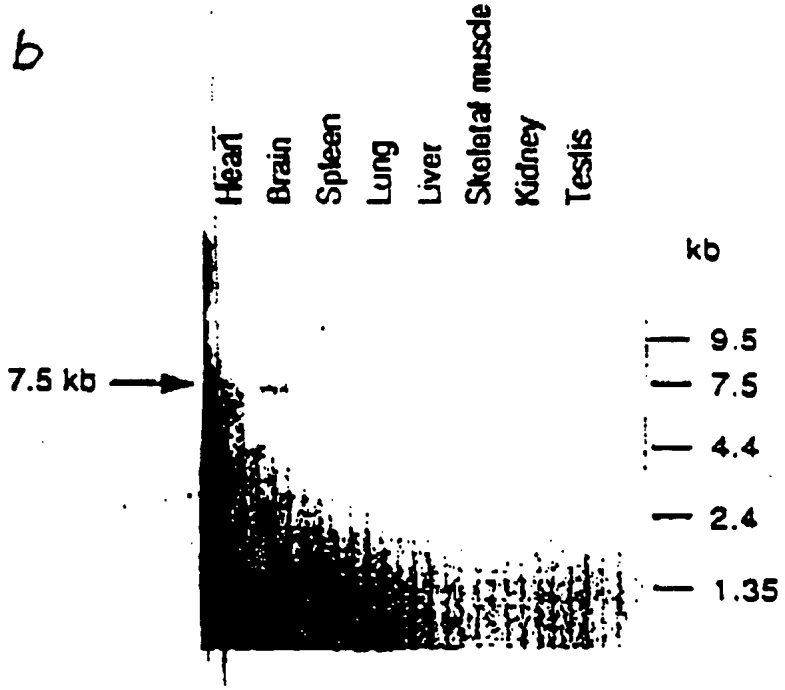
-1 (mouse)

```
GAGAGCACAGCCCTTCCCAGTCCCCGGAGCCGCCGCGCCACGCGCGCATGATCAAGACCTTTTCCCCGGCCCCACAGGGCCTCTGGACGT  90
GAGACCCCGGCCCGCCTCCGCAAGGAGAGGCGGGGGTCGAGTCGCCCTGTCCAAAGGCCTCAATCTAAACAATCTTGATTCCTGTTGCCGG 180
CTGGCGGGACCCTGAATGGCAGGAAATCTCACCACATCTCTTCTCCTATCTCCAAGGACCATGACCTTGGGGAGCTTCAGAAGGGGCGTT 870
                                                                  Met Thr Leu Gly Ser Phe Arg Arg Gly Leu

TTGATGCTGTCCGTGGCCTTGGGCCTAACCCAGGGGAGACTTGCGAAGCCTTCCAAGCTGGTGAACTGCACTTGTGAGAGCCCACACTGC 360
Leu Met Leu Ser Val Ala Leu Gly Leu Thr Gln Gly Arg Leu Ala Lys Pro Ser Lys Leu Val Asn Cys Thr Cys Glu Ser Pro His Cys

AAGAGACCATTCTGCCAGGGGTCATGGTGCACAGTGGTGCTGGTTCGAGAGCAGGGCAGGCACCCCCAGGTCTATCGGGGCTGTGGGAGC 460
Lys Arg Pro Phe Cys Gln Gly Ser Trp Cys Thr Val Val Leu Val Arg Glu Gln Gly Arg His Pro Gln Val Tyr Arg Gly Cys Gly Ser

CTGAACCAGGAGCTCTGCTTGGGACGTCCCACGGAGTTTCTGAACCATCACTGCTGCTATAGATCCTTCTGCAACCACAACGTGTCTCTG 540
Leu Asn Gln Glu Leu Cys Leu Gly Arg Pro Thr Glu Phe Leu Asn His His Cys Cys Tyr Arg Ser Phe Cys Asn His Asn Val Ser Leu

ATGCTGGAGGCCACCCAAACTCCTTCGGAGGAGCCAGAAGTTGATGCCCATCTGCCTCTGATCCTGGGTCCTGTGCTGGCCTTGCCGGTC 630
Met Leu Glu Ala Thr Gln Thr Pro Ser Glu Glu Pro Glu Val Asp Ala His Leu Pro Leu Ile Leu Gly Pro Val Leu Ala Leu Pro Val

CTGGTGGCCCTGGGTGCTCTGGGCTTGTGGCGTGTCCGGCGGAGGCAGGAGAAGCAGCGGGATTTGCACAGTGACCTGGGCGAGTCCAGT 720
Leu Val Ala Leu Gly Ala Leu Gly Leu Trp Arg Val Arg Arg Arg Gln Glu Lys Gln Arg Asp Leu His Ser Asp Leu Gly Glu Ser Ser

CTCATCCTGAAGGCATCTGAACAGGCAGACAGCATGTTGGGGGACTTCCTGGACAGCGACTGTACCACGGGCAGCGGCTCGCGGCTCCCC 810
Leu Ile Leu Lys Ala Ser Glu Gln Ala Asp Ser Met Leu Gly Asp Phe Leu Asp Ser Asp Cys Thr Thr Gly Ser Gly Ser Gly Leu Pro

TTCTTGGTGCAGAGGACGGTAGCTCGGCAGGTTGCGCTGGTAGAGTGTGTGGGAAAGGGCCGATATGGCGAGGTGTGGCCCCGGTTCGTGG 900
Phe Leu Val Gln Arg Thr Val Ala Arg Gln Val Ala Leu Val Glu Cys Val Gly Lys Gly Arg Tyr Gly Glu Val Trp Arg Gly Ser Trp

CATGGCGAAAGCGTGGCGGTCAAGATTTTCTCCTCACGAGATGAGCAGTCCTGGTTCCGGGAGACGGAGATCTACAACACAGTTCTGCTT 990
His Gly Glu Ser Val Ala Val Lys Ile Phe Ser Ser Arg Asp Glu Gln Ser Trp Phe Arg Glu Thr Glu Ile Tyr Asn Thr Val Leu Leu

AGACACGACAACATCCTACGCTTCATCGCCTCCGACATGACTTCGCCGAACTCGAGCACGCAGCTGTGGCTCATCACCCACTACCATGAA 1080
Arg His Asp Asn Ile Leu Gly Phe Ile Ala Ser Asp Met Thr Ser Arg Asn Ser Ser Thr Gln Leu Trp Leu Ile Thr His Tyr His Glu

CACGGCTCCCTCTATGACTTTCTGCAGAGGCAGACGCTGGAGCCCCAGTTGCTCCTGAGCTAGCTGTGTCCCCGGCCTGCCGCCTGGCG 1170
His Gly Ser Leu Tyr Asp Phe Leu Gln Arg Gln Thr Leu Glu Pro Gln Leu Ala Leu Arg Leu Ala Val Ser Pro Ala Cys Gly Leu Ala

CACCTACATGTGGAGATCTTTGGCACTCAAGGCAAACCAGCCATTGCCCATCGTGACCTCAAGAGTCGCAATGTGCTGGTCAAGAGTAAC 1260
His Leu His Val Glu Ile Phe Gly Thr Gln Gly Lys Pro Ala Ile Ala His Arg Asp Leu Lys Ser Arg Asn Val Leu Val Lys Ser Asn

TTGCAGTGTTGCATTGCAGACCTGGGACTGGCTGTGATGCACTCACAAAGCAACGAGTACCTGGATATCGGCAACACACCCCGAGTGGGT 1350
Leu Gln Cys Cys Ile Ala Asp Leu Gly Leu Ala Val Met His Ser Gln Ser Asn Glu Tyr Leu Asp Ile Gly Asn Thr Pro Arg Val Gly

ACCAAAAGATACATGGCACCCGAGGTCCTGGATGAGCACATCCGCACAGACTGCTTTGAGTCGTACAAGTGGACAGACATCTGGGCCTTT 1440
Thr Lys Arg Tyr Met Ala Pro Glu Val Leu Asp Glu His Ile Arg Thr Asp Cys Phe Glu Ser Tyr Lys Trp Thr Asp Ile Trp Ala Phe

GGCCTAGTGCTATGGGAGATCGCCCGCCGGACCATCATCAATGGCATTGTGGAGGATTACAGGCCACCTTTCTATGACATGGTACCCAAT 1530
Gly Leu Val Leu Trp Glu Ile Ala Arg Arg Thr Ile Ile Asn Gly Ile Val Glu Asp Tyr Arg Pro Pro Phe Tyr Asp Met Val Pro Asn

GACCCCAGTTTTGAGGACATGAAAAAGGTCGTGTGCGTTGACCAGCAGACACCCACCATCCCTAACCGGCTGCTGCAGATCCGGTCCTC 1620
Asp Pro Ser Phe Glu Asp Met Lys Lys Val Val Cys Val Asp Gln Gln Thr Pro Thr Ile Pro Asn Arg Leu Ala Ala Asp Pro Val Leu

TCCGGGCTGCCCCAGATGATGAGAGAGTGCTGGTACCCCAACCCCTCTGCTCCGCCTCACCGCACTGCGCATAAAGAAGACATTGCAGAAG 1710
Ser Gly Leu Ala Gln Met Met Arg Glu Cys Trp Tyr Pro Asn Pro Ser Ala Arg Leu Thr Ala Leu Arg Ile Lys Lys Thr Leu Gln Lys

CTCAGTCACAATCCAGAGAAGCCCAAAGTGATTCACTAGCCCAGCGCCACCAGGCTTCCTCTGCCTAAAGTGTGTGCTGGGGAAGAAGAC 1800
Leu Ser His Asn Pro Glu Lys Pro Lys Val Ile His *

ATAGCCTGTCTGCGTAGAGGGAGTGAAGAGAGTGTGCACGCTGCCCTGTGTGTGCCTGCTCAGCTTGCTCCCAGCCCATCCAGCCAAAAA 1890

TACAGCTGAGCTGAAATTCAAAAAAAAAAAAAA 1922
```

a



b


Heart
Brain
Spleen
Lung
Liver
Skeletal muscle
Kidney
Testis
kb
9.5
7.5
4.4
2.4
1.35
7.5 kb

c

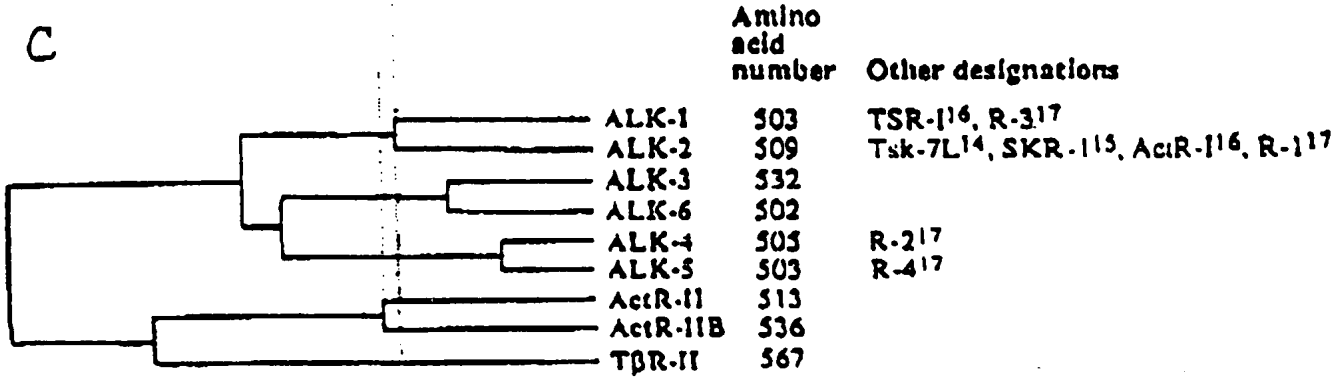
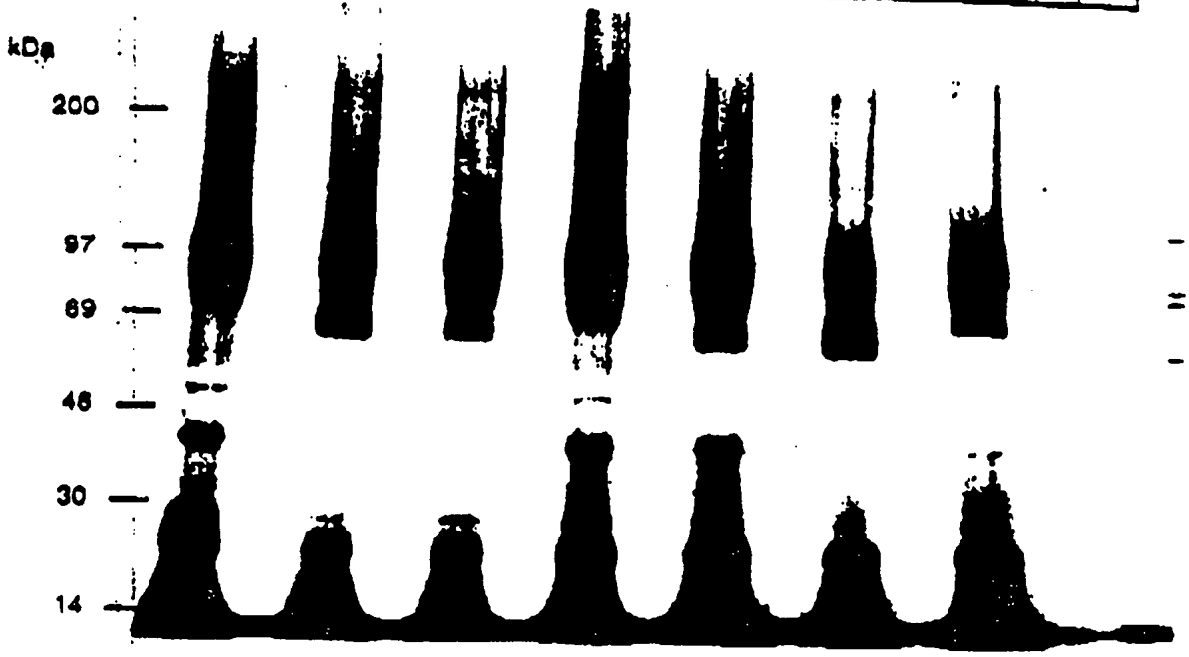

Amino acid number
Other designations
ALK-1 503 TSR-I[16], R-3[17]
ALK-2 509 Tsk-7L[14], SKR-1[15], ActR-I[16], R-1[17]
ALK-3 532
ALK-6 502
ALK-4 505 R-2[17]
ALK-5 503 R-4[17]
ActR-II 513
ActR-IIB 536
TβR-II 567

Figure 1.

a

TβR-II cDNA	+	+	+	+	+	+	+	-
ALK cDNA	-	ALK-1	ALK-2	ALK-3	ALK-4	ALK-5	ALK-6	-
cold TGF-β1	- +	- +	- +	- +	- +	- +	- +	-

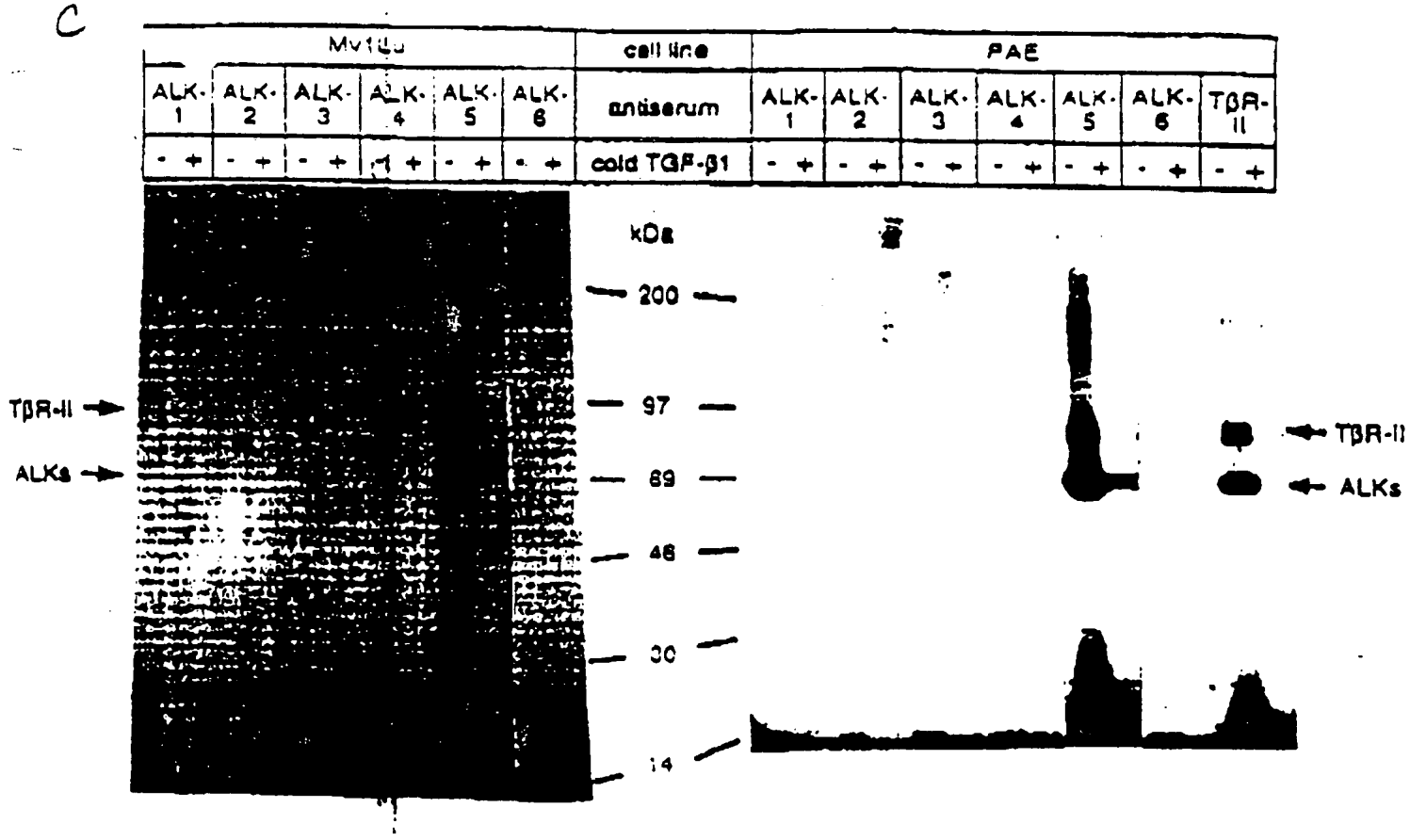


b

TβR-II cDNA	+						
ALK cDNA	ALK-1	ALK-2	ALK-3	ALK-4	ALK-5	ALK-6	-

Figure 2 a, b

c



d

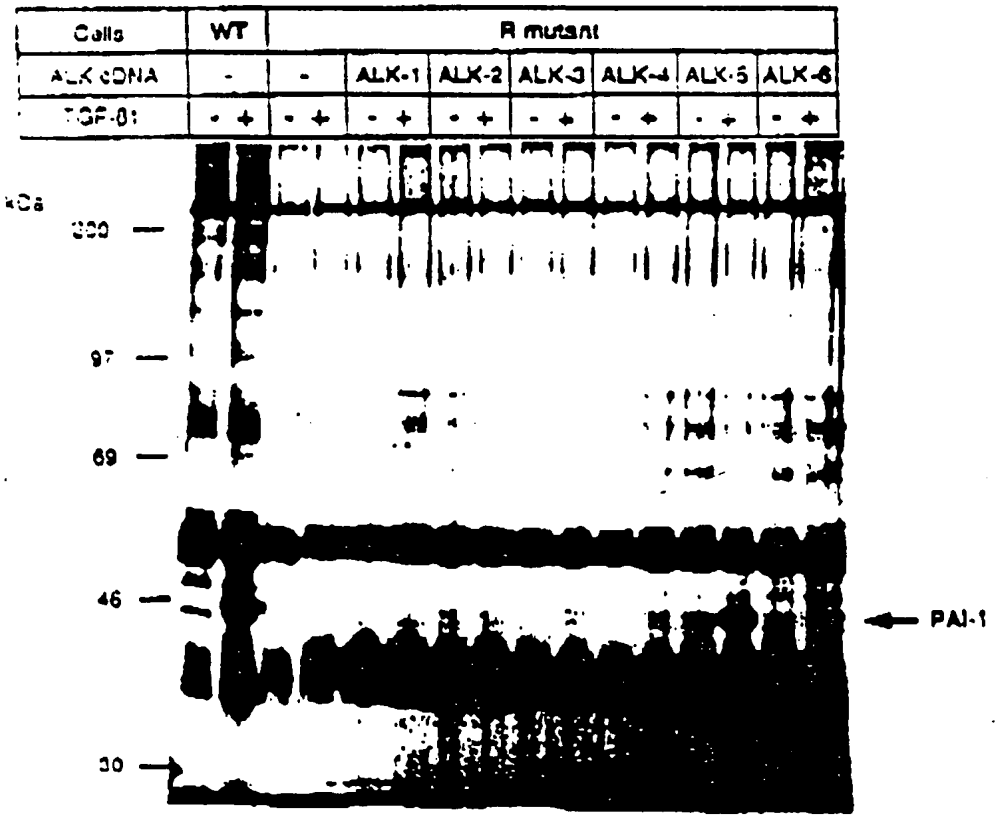
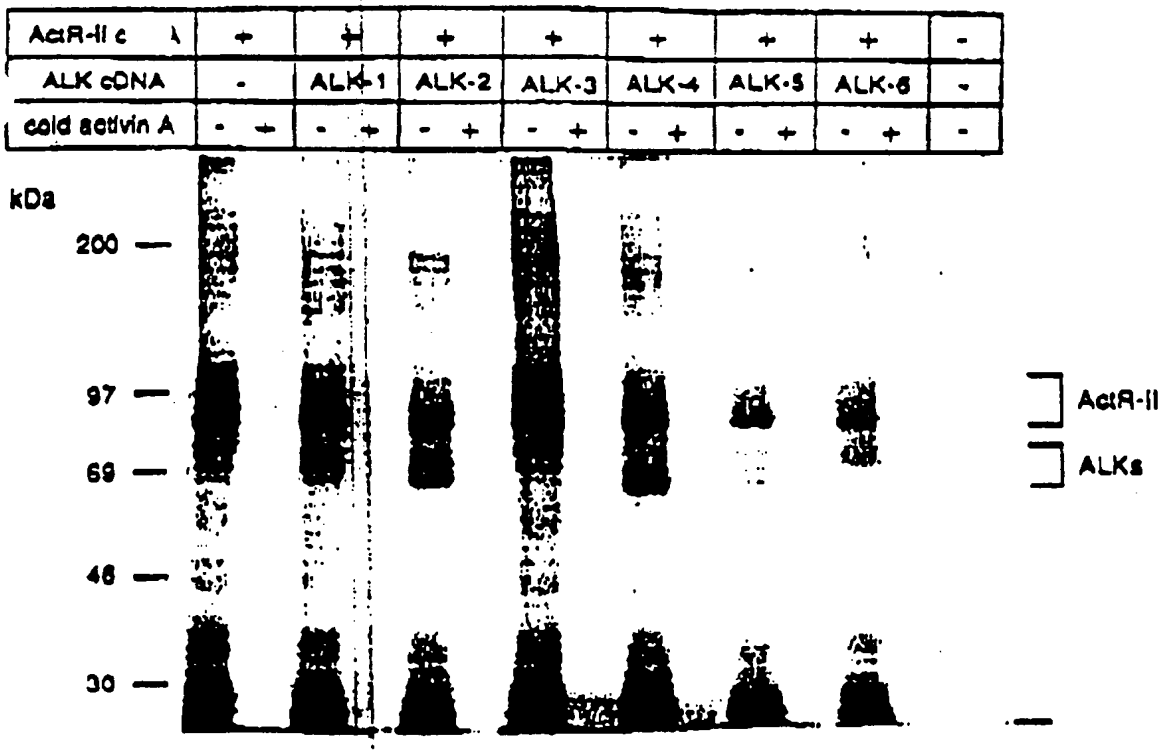


Figure 2c, d

a


ActR-II c
+ + + + + + + -
ALK cDNA
- ALK-1 ALK-2 ALK-3 ALK-4 ALK-5 ALK-6 -
cold activin A
- + - + - + - + - + - + -
kDa
200
97
69
46
30
ActR-II
ALKs

b

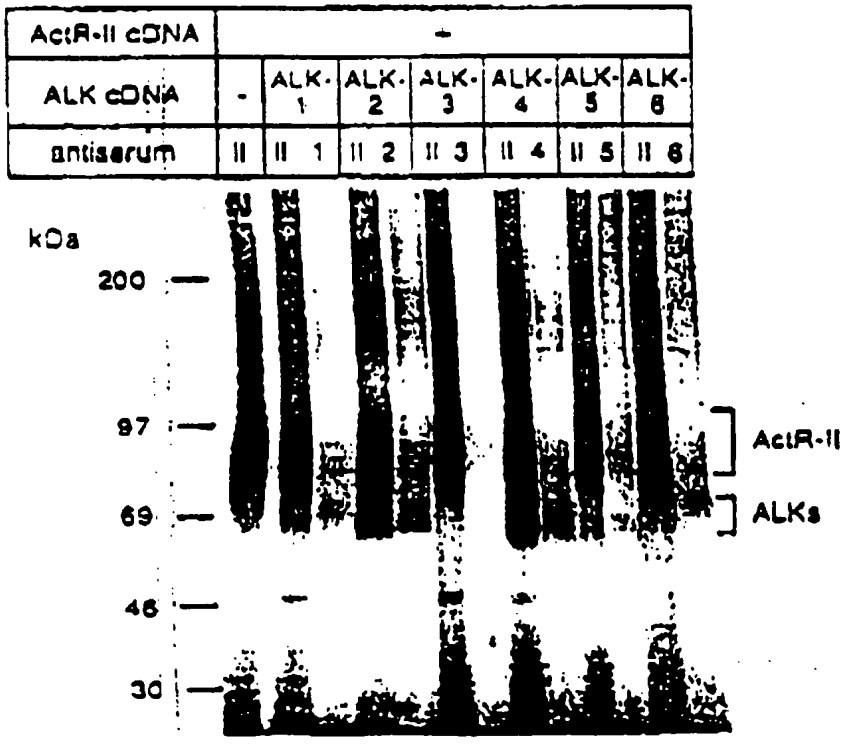

ActR-II cDNA
+
ALK cDNA
- ALK-1 ALK-2 ALK-3 ALK-4 ALK-5 ALK-6
antiserum
II II 1 II 2 II 3 II 4 II 5 II 6
kDa
200
97
69
46
30
ActR-II
ALKs

Figure 2b

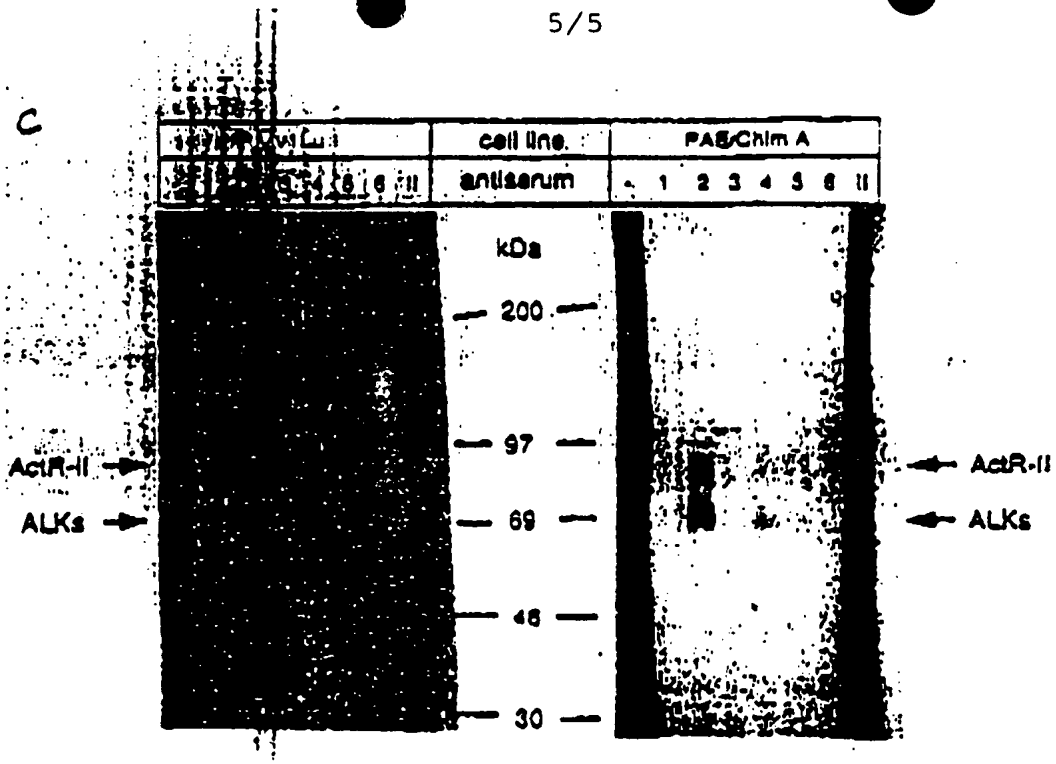


Figure 3C

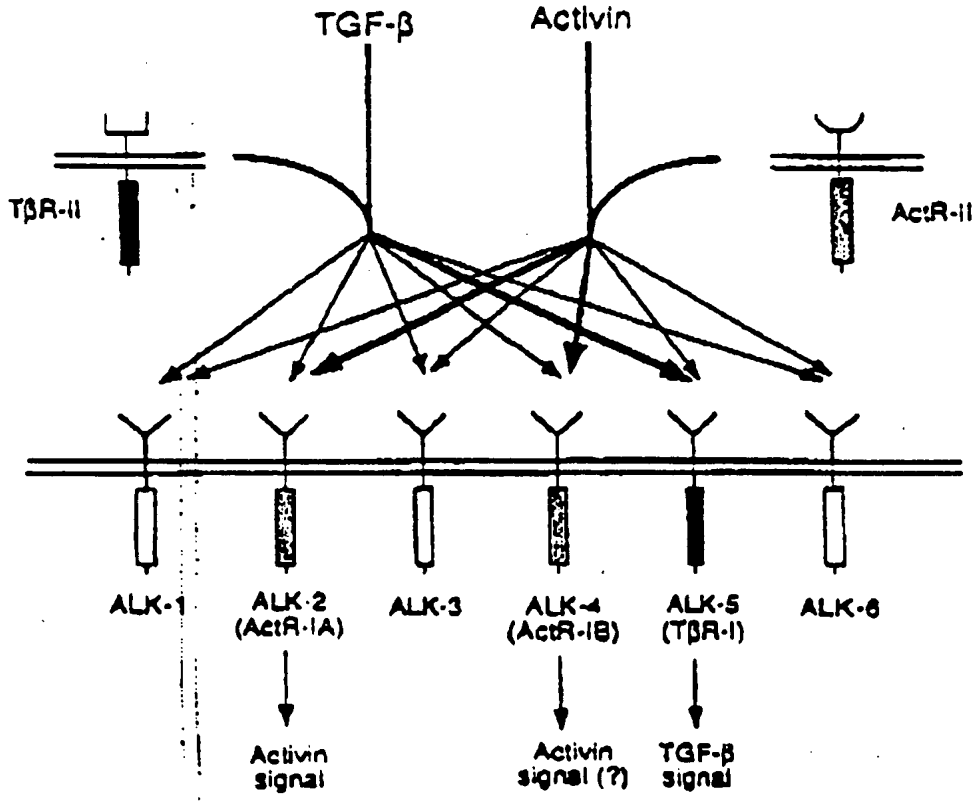


Figure 4